Modification of lysozyme by dry-heating: small cause, big effect

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Abbreviated title: Lysozyme dry-heating

Summary

Egg white dry-heating is usually achieved in industry to improve gelling and foaming properties. Major modifications of protein structure have been put forward to explain such functionality improvement. But as for lysozyme, one of the egg white proteins, dry-heating results in a great increase of foaming properties, without any significant modifications of structure, as determined thanks to usual analytical methods for secondary and tertiary structure determination. On the other hand, chemical modifications of the protein sequence could be highlighted for about 40% of lysozyme molecules: up to 5 Asp/Asn residues were changed for succinimide residues after dry-heating for 7 days at 80°C. These modifications result in more tensioactive forms, compared to native lysozyme. However, these "succinimide lysozymes" do not explain all the interfacial properties of dry-heated lysozyme. Indeed, the whole dry-heated lysozyme, that contains other modified forms supposed to be isoaspartate residues, do not have exactly the same behaviour. Finally, it can be concluded that lysozyme dry-heating induces several kind of modifications. And some of these modifications are different from structure changes usually mentioned to explain modification of protein properties. Anyway, dry-heated lysozyme is actually a very efficient foaming agent, contrary to the native one.

Keywords: lysozyme, dry-heating, structure, interfacial properties, foaming properties

Introduction

The mechanisms of formation and stabilization of foams are of particular interest for food manufacturers. Proteins are the most widely used stabilizers in the food industry but they often cannot provide sufficient long-term foam stability due to drainage, coalescence and disproportionation. Amongst the different methods developed to improve protein functionality, dry-heating seems to be the most promising approach, especially for egg white (Kato et al., 1989). First, it is very efficient to improve foaming properties, without a significant loss of solubility; second, it can be used easily in an industrial context and provides ready-to-use food powders. However, the modifications induced by such a treatment and responsible for the improvement of foaming properties are not fully elucidated (Kato et al., 1990; Matsudomi et al., 2001). Especially, the usual assumption of tertiary and secondary structure modifications, explaining a more efficient position of the proteins at the air-water interface, is not consistent with the present experimental results. To progress in the understanding of the modifications induced by dry-heating of egg white, lysozyme has been here chosen as a model protein. Indeed, this small (129 amino acids), rigid (4 disulfide bridges) and poorly hydrophobic protein exhibits very low interfacial properties when it is in native form, while dry-heating makes it a very efficient foaming agent (Desfougères et al., 2008). The present study aims to identify which modifications occur on lysozyme during heat-treatment, and could explain the strong improvement of its foaming properties.

Materials and Methods

Spray-dried hydrochloric lysozyme was supplied by Ovonor (Annezin, 62-France). The powder contained 7% H_2O, corresponding to a water activity of 0.31± 0.02. After dissolution in water, the pH of lysozyme solution was 3.5. To perform dry-heating treatment, lysozyme powder was placed in hermetically capped glass tubes (3-5g in each) and stored in an oven at 80°C for 0 to 7 days. After treatment, it was kept at 4°C until use. Lysozyme was dissolved in 60mM phosphate buffer pH 7.0 (μ=140mM) before performing the different experiments described below.
Foaming properties were analysed as described by Baniel et al. (1997), using a bubbling column PM930 (Grosseron, St. Herblain, France). Compressed air was injected at a constant flow rate (25mL.min^{-1}) in 12mL of sample through a porous metallic disk placed at the bottom of the column. Bubbling was stopped when the foam volume reached 50mL (V). The evolution of the foam was then recorded for 30min. Conductivity measurements were used to calculate the

volume of liquid in the foam and thus allowed to characterize the foam density (FD) and the foam stability (FS). These 2 parameters were calculated as follows:

$$FD = V_m/V \qquad FS = V_f/V_m \times 100,$$

where V_m is the maximum volume of liquid incorporated in the foam, and

V_f is the volume of liquid remaining in the foam after 30min.

The ellipsometric measurements were carried out with a conventional null ellipsometer using a He–Ne laser operating at 632.8nm (Berge and Renault, 1993). The ellipsometric angle (δ) and surface pressure (π) were recorded simultaneously. The surface pressure was measured with the Wilhelmy film balance. The volume of the Teflons sample trough was 8mL. Eighty microliters of lysozyme prepared at 10mg.mL^{-1} in H_2O were injected in sub-phase of 8 mL of 60mM phosphate buffer pH 7.0 using a Hamilton syringe; final protein concentration was 0.1mg.mL^{-1}. All the experiments were performed at room temperature in the range 19–21°C.

Cation exchange-HPLC was performed on a S-HyperD10 column (10µm, Biosepra, Cergy Saint-Christophe, France). Elution was achieved using a NaCl gradient from 0 to 1M in 20mM acetate buffer pH 5.0. Protein detection was carried out by spectrophotometry at 280nm.

Measurement of surface hydrophobicity was carried out using the fluorescent probe ANS. Fifteen microliters of an 8mM ANS solution were added to 1mL of the protein sample solution. Then, fluorescence intensity was measured between 420 and 560nm, after excitation at 390nm.

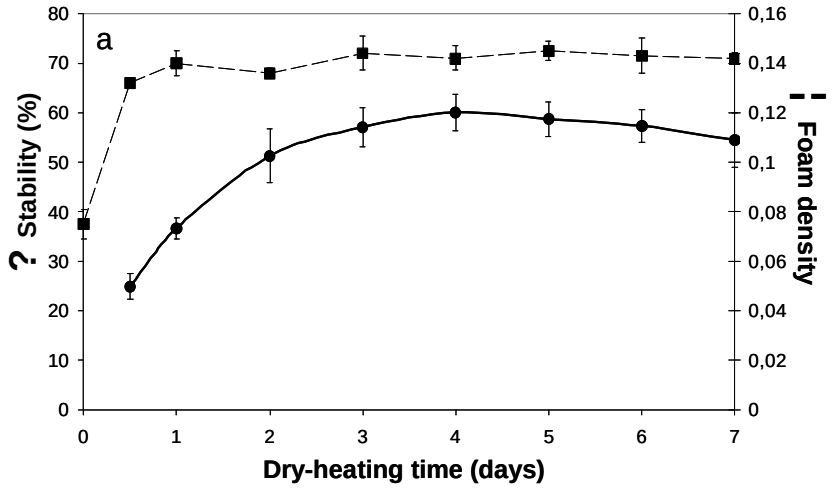
Lysozyme mass analyses were achieved using a Q $STAR_{XL}$ MS/MS system equipped with a nanoESI source (Applied Biosystems). Peptides sequencing was performed by LC/MS/MS after digestion by pepsin and reduction with TCEP (Tris(2-carboxyethyl)phosphine HCl). Liquid chromatography was achieved with a nanoHPLC system, equipped with a C_{18} PepMap column (3µm, 100Å, 7.5µm i.d. x 150mm), using an acetonitrile gradient in a 0.08% HCOOH/ 0.01% TFA buffer.

Results and discussion

Dry-heating makes lysozyme an efficient foaming agent

Native lysozyme is a very poor foaming agent, especially at neutral pH, meaning far from its pI. But this protein exhibits excellent foaming properties after dry-heating. In the conditions applied in the present study, the optimal properties are observed after only 2 days heating (Figure 1a): foam density is strongly increased (x 1.8), as well as foam stability (x 2.4). Then, the same stability is obtained with a 20mg.mL^{-1} native lysozyme and 5mg.mL^{-1} lysozyme dry-heated for 0.5 day or 4mg.mL^{-1} lysozyme dry-heated for 1 day (Figure 1b).

Figure 1. Foaming properties of native and dry-heated lysozyme: (a) foam density and stability vs dry-heating treatment duration (protein concentration of 10mg.mL^{-1}); (b) foam stability vs lysozyme concentration.



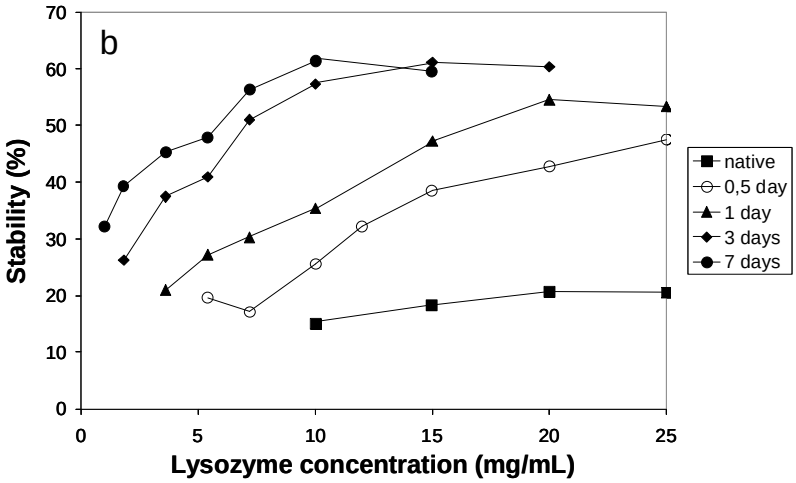
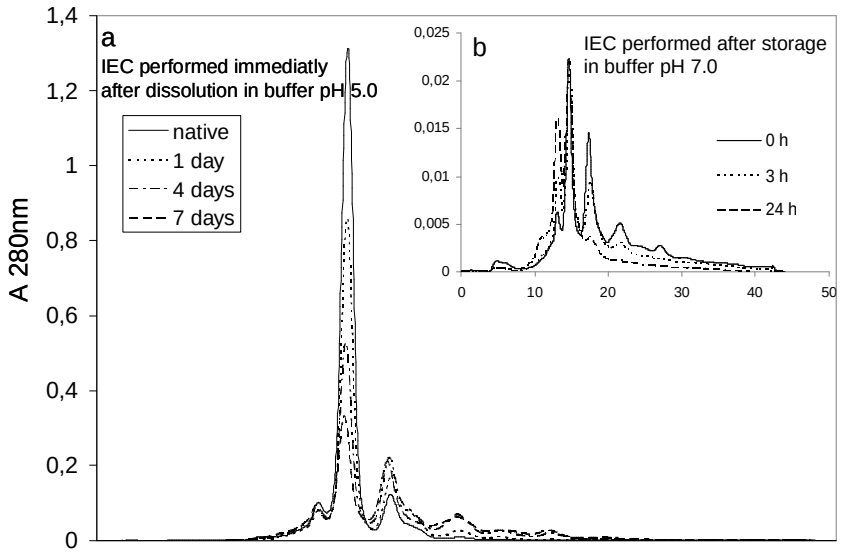


Figure 2. Cation exchange chromatograms: (a) native and dry-heated lysozymes analysed immediately after dissolution in buffer pH 5.0; (b) 7 days heat-treated lysozyme analysed after storage in buffer pH 7.0.

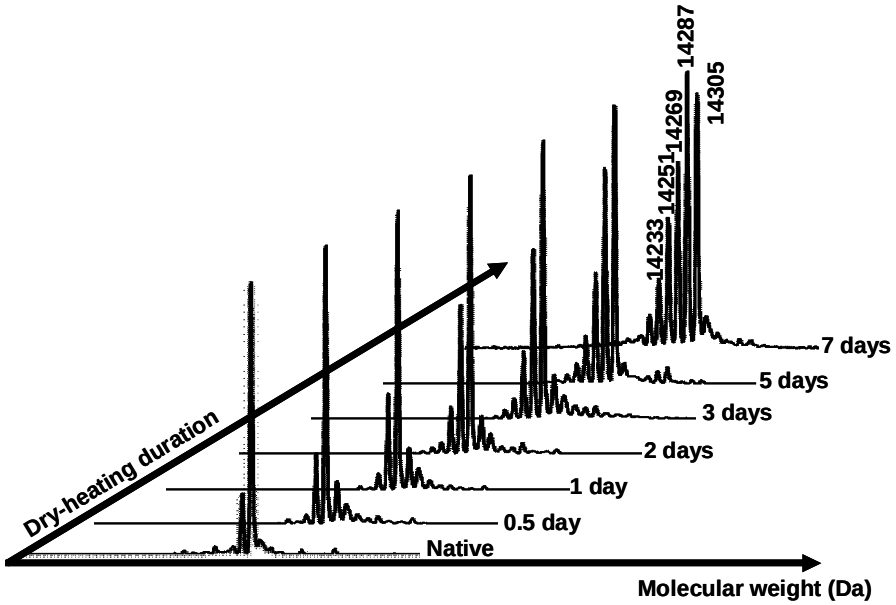


Which kind of modifications induced on lysozyme molecule ?

Ion (cation) exchange chromatography (IEC) performed at pH 5.0 reveals significant modifications induced by dry-heating. As dry-heating duration increases, peaks more and more higher appear at retention times longer than this of native lysozyme; these peaks reflect then the formation of molecular species more alkalin than native lysozyme. At the same time, the native-lysozyme peak decreases (Figure 2a). It is noticeable that when dry-heated lysozyme solution is stored at pH 7.0, and analysed at this slightly basic pH, these more alkalin molecular species progressively disappear, while molecular forms appear at retention time shorter than this of native lysozyme; these new molecules are then less alkalin than the native protein (Figure 2b). These results highlight that dry-heating induces surface charge modifications, resulting in several molecular species, some of them being sensitive to pH. On the other hand, dry-heating induces formation of molecules with molecular weights weaker than this of native lysozyme; up to 5 species can be detected by mass spectrometry analysis, all distant of 18Da from each other (Figure 3). To identify which peaks obtained by IEC correspond to those lighter lysozymes, all of them have been collected and submitted to mass spectrometry analysis. For the major peak, meaning with same retention time than native lysozyme, the experimental molecular weight was identical to the theoretical one of lysozyme, that is 14305Da. The same was true for the peaks corresponding to shorter retention times than native lysozyme. On the contrary, the first peak eluted after native lysozyme corresponded to a molecular weight of 14287Da, meaning 18Da less than native lsyozyme. And when collected together, the species eluted after native lysozyme corresponded to a mixture of 4 different lighter

lysozymes, that is with molecular weights of 14287, 14269, 14251, and 14233Da, respectively (data not shown). The most alkalin species of lysozyme are then chemically different from native lysozyme. However, not any difference could be observed by circular dichroism between, on the one hand, native lysozyme and the less alkalin species, and on the other hand, the more alkalin species collected all together (data not shown). This result suggests that, despite dry-heating induces chemical modifications, it does not imply any significant change of secondary and/or tertiary structure of lysozyme.

Figure 3. Dry-heating of lysozyme induces formation of molecular species 18Da away from each other, as determined by mass spectrometry analysis.



Dry-heating of lysozyme induces succinimide and isoaspartate formation

As it was already described for different proteins, and especially for heated lysozyme solutions (Tomizawa et al., 1994), the results obtained here suggest that dry-heating of lysozyme induces modification of Asp/Asn residues into succinimide (Figure 4). Indeed, such a modification comes with a loss of 18Da. Moreover, it is well established that succinimyl cycles are favored at acidic pH, but hydrolysed at pH higher than 6.0, to be changed into Asp or IsoAsp, in a ratio equal to about 75/25 (IsoAsp/Asp). Then, the disappearance of the "-18Da forms" (higher retention time in IEC), when dry-heated lysozyme is stored at pH 7.0, is consistent with the assumption of succinimide formation. Finally, because succinimides are more hydrophobic groups than Asp and Asn residues, it could explain the higher surface

hydrophobicity of the "-18Da lysozymes" (Figure 5). Finally, the Asp/Asn concerned by the modification have been identified. In that aim, LC/MS/MS has been achieved on pepsic digests obtained from either native lysozyme, or "-18Da lysozymes", after reduction at pH 5.0 by TCEP. Then, the peptides for which differences of 18Da or 36Da exist between the both kinds of lysozyme have been sequenced; all of them contained Asp/Asn residues. During sequencing, some Asp/Asn residues have been clearly identified, in that mass analysis enables to state positively it is either Asp/Asn residues, or succinimide residues. But mass analysis data were not always so clear. Then, the most likely residues involved in succinimide formation have been finally determined, according to the probability rules of such a modification; indeed, depending on the amino acid just following the Asp/Asn residue, succinimide formation is favoured or unfavoured; moreover, some flexibility of the carbon chain is required to enable succinimide formation (Robinson and Robinson, 2001; Robinson, 2002). The 5 most likely Asp/Asn residues involved in succinimide formation during dry-heating of lysozyme are resumed in Table 1. It can be noticed that Asp101, already described as a potential modification site on lysozyme (Yamada et al., 1985; Tomizawa et al., 1994), as well as Asp18, seem to be the most sensitive amino acids, since they are those modified when only one 18Da mass is lost from lysozyme molecule.. As far as IsoAsp, it could be the modified lysozyme with lower retention time than native one, since it appears when pH is slightly basic. But no demonstration could be done in the present study, because no mass difference exists between Asp and IsoAsp.

Figure 4. Simplified reactional schema of protein deamidation and isomerisation.

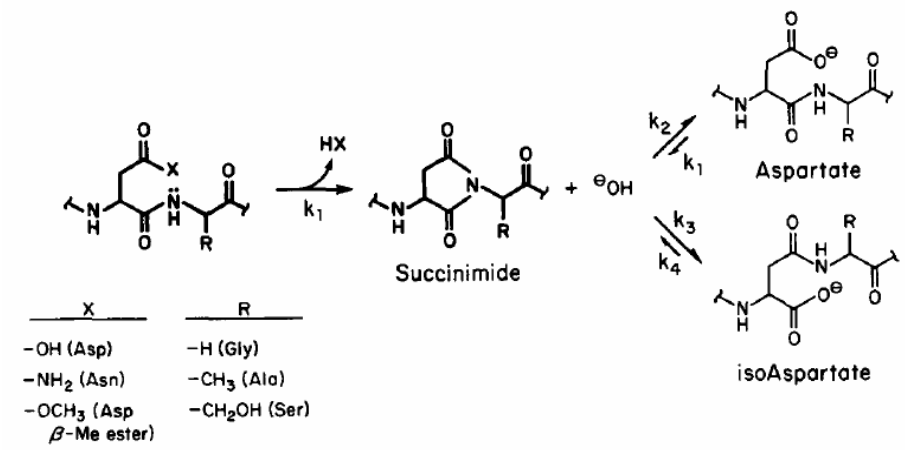


Figure 5. Surface hydrophobicity of native, dry-heated and succinimide lysozymes, as detremined by fluorescence intensity after ANS addition to protein solution and excitation at 390nm.

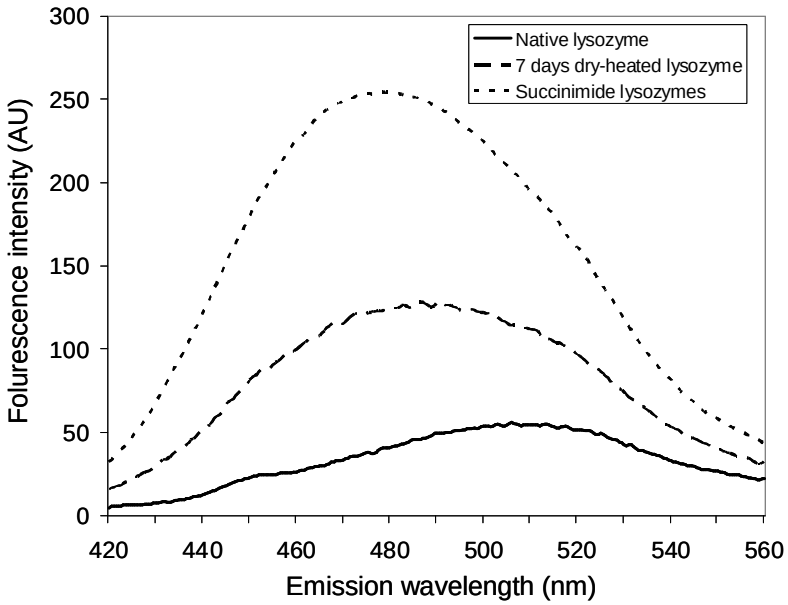
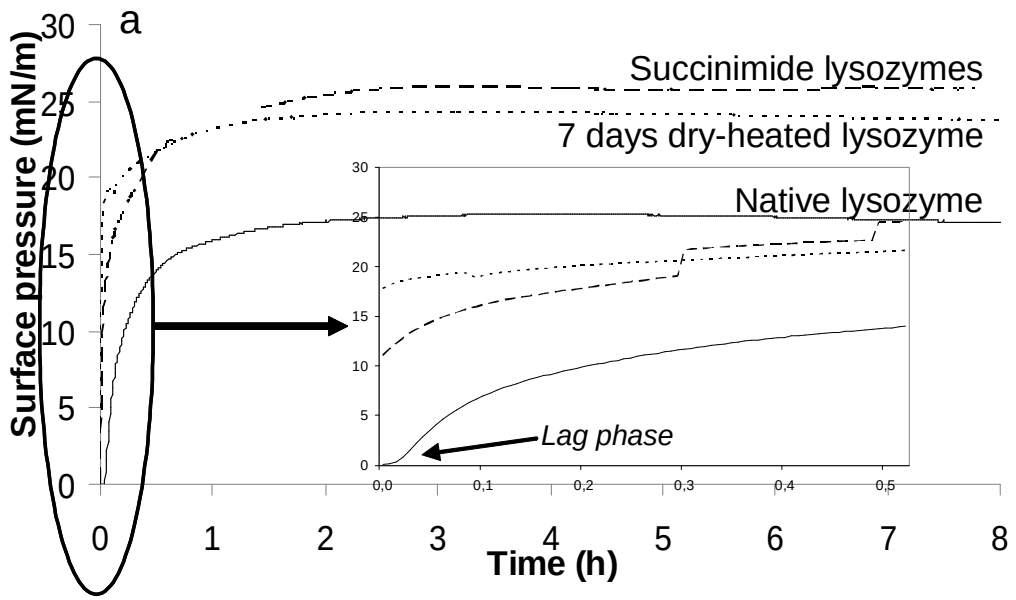


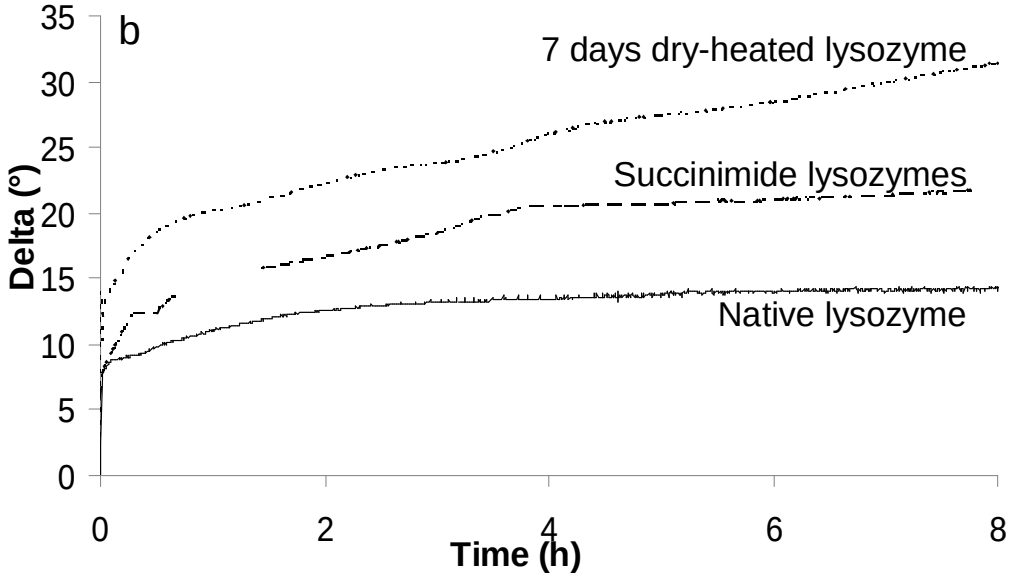
Table 1. Identification of the amino acid residues changed in succinimide residues due to dry-heating of lysozyme. Residues certainly identified as modified are indicated in bold and bigger characters; residues certainly identified as non modified are indicated in normal characters; residues for which identification is not definite are underlined and in bold, and the most likely aminoacids concerned by succinimide formation are proposed, according to the following probability rules: Asn44 is in a β-sheet and followed by Arg, then very unlikely; Asp52 is in a β-sheet and followed by Tyr, then very unlikely; Asn74 and Asn77 are in a random coil but followed by Leu and Ile, respectively, then very unlikely).

Peptides	Sequences	Δ Masses (Theo.-Obs.)	Scores Mascot*	Identified or most likely aminoacids
9-22	AAAMKRHGLDNYRG	18 Da	35,2	Asp18
39-53	NTQATNRLTDGSTDY	18 Da	84,0	Asp48
39-56	NTQATNRLTDGSTDYGIL	18 Da	29,9	Asp48
63-83	WCNDGRTPGSRNLCNIPCSAL	18 Da	37,1	Asn65 or Asp66
91-107	SVNCAKKIVSDGNGMNA	36 Da	49,3	Asp101, Asn103
95-108	AKKIVSDGNGMNAW	36 Da	37,1	Asp101, Asn103

*score > 28 means $p < 0.05$

Figure 6. Interfacial properties of native, dry-heated and succinimide lysozymes (protein concentration in the bulk is 0.1mg.mL^{-1} in 60mM phosphate buffer pH7.0, μ=140mM): (a) surface pressure and (b) ellipsometric angle.





Without secondary and tertiary structure modifications, chemical modifications induced by dry-heating strongly improve interfacial behaviour of lysozyme

Especially, dry-heated lysozyme does not exhibit any lag phase before interfacial adsorption, contrary to the native one, and final surface pressure is much higher (Figure 6a). Moreover, while native lysozyme creates a defined and thin interfacial layer, whole dry-heated and succinimide lysozymes produce much more thick layers (Figure 6b); thickness of the interfacial layer could be estimated by atomic force microscopy analysis, equal to 2nm, 80-100nm and 70-80nm, respectively. These results could be related to the aggregation ability of dry-heated lysozyme, previously described (Desfougères et al., 2008). Its higher affinity for air-water interface, plus its ability to create a strong and thick interfacial layer could finally explain the much higher foaming properties of dry-heated lysozyme, meaning higher foam density and stability.

One of the most noticeable results of this study concerns the demonstration that higher foaming properties of a protein do not systematically imply modifications of its secondary or tertiary structure. Our hypothesis consists in that some punctual chemical modifications due to treatment can induce higher flexibility of the protein molecules. And this higher flexibility enables that the protein denatures more efficiently at the air-water interface, and becomes able to strongly interact with each others, to create a very stable interfacial layer. This assumption is consistent with the recent results obtained by Perriman et al. (2008) who observed lysozyme aggregation and multilayer formation at air-water interface when lysozyme is previously denatured by guanidinium chloride.

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